WEST

Generate Collection Print

L2: Entry 5 of 6 File: USPT Oct 10, 2000

DOCUMENT-IDENTIFIER: US 6130092 A
TITLE: Ribozyme gene library and method for making

Abstract Text (1):
A ribozyme library which comprises a collection of ribozyme genes encoding a hammerhead structure and flanking sequences of random nucleotides cloned at least once into an expression cassette for ribozyme expression.

Priority Application Year (1):
1994

Priority Application Year (2):
1994

Brief Summary Text (2):
The present invention relates to a ribozyme library, its preparation and use. The library of the present invention contains ribozymes for desired target sequences for use in the fields of molecular biology, genetic engineering, and in medicine, by expression and in vivo and in vitro insertion, suitably for identification and switching off genes in the case of diseased conditions.

Brief Summary Text (4):
The inactivation of gene functions by reverse genetic material is the most important method for switching off certain genes. This is of great importance for combating infectious and other diseases, including AIDS, caused by interference with gene expression. A gene function can be inactivated on various levels, by homologous recombination at the DNA level, by antisense nucleic acids or ribozymes on the RNA level, or by antibodies on the protein level. In conversion to practice, all of these possibilities have advantages and disadvantages. For therapeutic applications, only the RNA inactivation by antisense molecules or by ribozymes appears to be implementable. Both classes of compounds can be synthesized chemically or produced in conjunction with a promoter by biological expression in vitro or even in vivo. The principle of catalytic self-cleavage of RNA molecules and the cleavage in trans has become well established in the last 10 years. The hammerhead ribozymes are characterized best among the RNA molecules with ribozyme activity. Since it was shown that hammerhead structures can be integrated into heterologous RNA sequences and that ribozyme activity can thereby be transferred to these molecules, it seems clear that catalytic antisense sequences for almost any target sequence can be created provided the target sequence contains a potential cleavage site.

Brief Summary Text (5):
The basic principle of constructing ribozymes is quite simple. An interesting region of the RNA, which contains the GUC (or CUC) triplet, is selected. Two oligonucleotide strands, each with 6 to 8 nucleotides, are taken and the catalytic hammerhead sequence is inserted between them.

Brief Summary Text (6):
Molecules of this type were synthesized for numerous target sequences. They showed catalytic activity in vitro and in some cases also in vivo. The best results were obtained with short ribozymes and target sequences. A topical challenge for the in vivo application is the construction of ribozyme genes which permit a continuous expression of the ribozyme in a particular cell (Bertrand, E. et al., (1994) Nucleic

Acids Res. 22, 293 to 300).

Brief Summary Text (7):
There are five possible causes for interference with a satisfactory functioning of expressed ribozymes within the complex intracellular milieu:

Brief Summary Text (8):
1. The mRNA substrate exists within the cell presumably in a highly folded structure, which can also be protected by proteins bound to parts of the structure. The encountering of accessible sites within the substrate allowing for hybridization with the complementary flanking regions of the ribozyme, is a question of actual probability. Computer aided predictions of possible, thermodynamically stable secondary structures can be useful when searching for loop regions without base pairing. However, the physiological relevance of these conformation models is still uncertain.

Brief Summary Text (9):
2. Since the target mRNA is transported immediately out of the cell nucleus, the ribozyme must also enter the cytoplasm, preferably along the same path. It is, however, difficult to achieve a co-localization of ribozyme and its substrate.

Brief Summary Text (10):
3. The in vivo use of ribozymes requires the insertion of ribozyme genes in suitable expression cassettes. The transcription of these constructs can produce mRNAs, in which the central, catalytic, secondary structure of the ribozymes is displaced by other, more stable base pairings within the non-complementary flanking sequences. Suitable expression cassettes can be constructed in accordance with the prior art, to express the ribozyme library.

Brief Summary Text (11):
4. A 100- to 1,000-fold excess of ribozyme molecules relative to the target sequence is necessary, for attaining a recordable increase in the RNA level. The production of 105 to 106 ribozymes per cell over a long period of time can, however, have cytotoxic effects. In general, such high expression levels are not stable. An excess of ribozymes is needed because of the inadequate stability of the ribozymes in the presence of nucleases, because of the ineffective transport to the cytoplasm and because of the less than optimum conversion factor of the cleavage reaction.

Brief Summary Text (12):
5. The kinetics of the cleavage reaction and the ability of the ribozymes to carry out multiple conversion reactions depends on the binding parameters and the structure of the complementary flanking regions of the ribozymes. Cellular proteins can affect the catalysis of the cleavage reaction, probably with the help of the dissociation of the ribozyme from the substrate of the cleavage reaction, which represents the preliminary step of the next cleavage reaction. Until now, it has not been possible to predict the optimum structure of the flanking regions for a ribozyme, to guarantee high specificity and high conversion. It can be noted that, despite many efforts to construct specific ribozyme genes, generally only partial successes have been achieved, mostly on the basis of trial and error experimentation. Expression cassettes useful in conjunction with the present invention and their preparation, are also described in:

Brief Summary Text (13):
Cameron F. H. and Jennings. P. A. (1989). Specific gene suppression by engineered ribozymes in monkey cells Proc. Natl. Acad. Sci. USA 86. 9139-9143.

Brief Summary Text (14):
Cotten M. and Birnstiel. M. L. (1989). Ribozyme-medicated destruction of RNA in vivo. EMBO J.8, 3861-3866.

Brief Summary Text (15):
Efiat s. Leiser. M. Wu. Y. J. Fusco-DeMane, D. Emran, O. A., Surana. M. Jetton. T. L., Magnuson, M. A. Weir C. and Fleischer, N (1994) Ribozyme-medicated attenuation of pancreatic B-cell glucokinase expression in transgenic mice results in impaired glucose-induced insulin secretion Proc. Natl. Acad. Sci. USA 91, 2051-2055.

Brief Summary Text (16):
Rossi J. J (1995), Controlled, targeted, intracellular expression of ribozyme; progress and problems. TIBTECH 13. 301-306.

Brief Summary Text (17):
Sioud M. and Drlica. K (1991). Prevention of human immunodeficiency virus type 1 integrase expression in Eschenchia Coli by a ribozyme. Proc. Natl. Acad. Sci. USA 88, 7303-7307.

Brief Summary Text (18):
Shore, S. K., Nabissa, P. M. and Reddy, E. P. (1993) Ribozyme-medicated

Brief Summary Text (20):
Steinecke, P. Herget. T. and Schreter, P. H. (1992). Expression of a chimeric ribozyme gene results in endonucleolytic cleavage of target mRNA and a concomitant reduction of gene expression in vivo EMBO J. 11. 1525-1530.

Brief Summary Text (21):
Thompson. J. D. Ayers, D. F. Malmstrom. T. A. McKenzie T. L. Ganousis. L. Chowrira. B. M. Couture, I, and Stinchcomb, D. T. (1995). Improved accumulation and activity of ribozymes expressed from a tRNA-based RNA polymerase III promoter. Nucleic Acids Res. 23. 2259-2268.

Brief Summary Text (23):
It is an object of the present invention to enable preparation of optimum ribozymes for any target sequence. It enables the effective expression of stable ribozymes, suitably for identifying and switching off genes in the case of illnesses.

Brief Summary Text (24):
The present invention is a ribozyme gene library, and a method for producing the ribozyme gene library wherein the desired target sequence, or that one that is to be switched off, itself searches out the most suitable ribozyme from a selection of ribozymes with known stability and structure. Pursuant to the present invention, this is accomplished due to the ribozyme library which can be expressed within any expression cassette. For example one can use a conventional polymerase II-promoter (e.g. cytomegalovirus CMV), a piece of a gene coding for mRNA, as well as a termination sequence. Another way of expressing the ribozyme library is a conventional polymerase I-gene with a promoter and a termination sequence. Yet another way is a polymerase III-gene with an internal promoter (e.g. the VA-RNA gene).

Brief Summary Text (25):
However, the biological activity of the ribozyme requires a secondary structure formation which is correctly formed on one side of the ribozyme structure, and on the other side prevents the flanking DNA sequences from a structural inhibition of the ribozyme activity. The conventional expression cassettes of the three above-exemplified types enable the expression of all ribozyme genes in the library, but they can have an unpredictable effect on the folding of individual ribozyme-RNAs. An expression cassette can be optimized, containing from about 10.sup.9 to about 10.sup.11 ribozyme genes, and the expression cassette suitably contains a T7 promoter, an adenoviral va-RNA-gene, and a stable loop region to assure an open structure of the ribozyme sequences. These ribozymes have a central hammerhead structure of defined sequence and flanking sequences of bases arranged randomly. The hammerhead structure is coded by a double stranded gene, in which the hammerhead is enclosed on both sides by flanking, random sequences of bases.

Brief Summary Text (26):
An analogous library can also be created with other antisense genes.

Drawing Description Text (3):
FIG. 1 shows the construction of the ribozyme library of the present invention (SEQ ID NO: 5,6);

Drawing Description Text (4):
FIG. 2 shows the use of the ribozyme library of the present invention (SEQ ID NO: 5,

7, 8);

Drawing Description Text (5):
FIG. 3 shows amplification and cloning of ribozyme specific for the human growth hormone (hGH) gene (SEQ ID NO: 9);

Drawing Description Text (6):
FIG. 4 is the partial nucleotide sequence of the hGH gene;

Drawing Description Text (9):
FIGS. 7A-C show the result of cleavage of hGH RNA in vitro by a ribozyme. More specifically FIG. 7(A) shows the structure of ribozyme (SEQ ID NO: 12);

Drawing Description Text (10):
FIG. 7(B) shows plasmid matrices for ribozyme synthesis; and

Detailed Description Text (2):
The double-stranded hammerhead region suitably has the sequence CTGATGAGTCCGTGAGGACGAAAC (Seq. Id. No. 1) and the flanking sequences suitably have a length of from about 6 to about 13 nucleotides. The construction of the ribozyme library of the present invention, as shown in FIG. 1, starts out from synthetic oligonucleotides with a random sequence of from about 6 to about 13 nucleotides. These are combined with the ribozyme sequence, converted into a double strand and cloned with flanking restriction sites into the corresponding insertion site of the expression cassette.

Detailed Description Text (3):
In explaining the diagrammatic showing of the use of the ribozyme library of the present invention in FIG. 2, growth hormone can be used as an example. The growth hormone gene has 150 theoretical cleavage sites (ribozyme binding sites, GUC or CUC base sequences). Only some cleavage sites will be accessible in vivo. These and the most effective ribozymes, matching the cleavage sites, are to be isolated.

Detailed Description Text (4):
First a pool of ribozyme genes is synthetically produced for this purpose, a central "hammerhead" ribozyme sequence being flanked by 13 nucleotides of random sequence. These genes are cloned into an expression vector (GvaL) for ribozymes into a sequence coding for the adenoviral va-RNA, allowing for high stability, which is under the control of a T7 promoter, and flanked on both sides by an identical sequence of 21 nucleotides, which are to prevent the formation of a secondary structure. The cloning results in a library of about 10.sup.9 clones. To isolate a specific ribozyme, the target gene is transcribed in vitro from a suitable construct (with T7-polymerase or RNA polymerase III) and the RNA obtained is incubated with the ribozyme library, which was also transcribed in vitro. Subsequently, the cleavage products are separated electrophoretically. Clearly identifiable fragments are extracted from the gel and sequenced.

Detailed Description Text (5):
The sequence at the ends of the fragments permits the cleavage site to be defined and the ribozyme, responsible for the cleavage site, to be identified. The ribozyme in question is amplified from the ribozyme library using two oligonucleotides specific for its flanking sequences, and cloned once again in the vector GvaL as described below in Example 1. The ribozyme activity of the library is detected by incubation with total cellular or cytoplasmic RNA and its degradation, as described below in Example 2. The presence of ribozymes against a particular target RNA, such as hGH, is detected by incubation with an in vitro transcribed RNA, as described below in Example 3. The cleavage sites are localized by isolating fragments of the cleaved target RNA and sequencing them, as described below in Example 4. The isolation of specific ribozymes is carried out by hybridization with oligonucleotides that are specific for the flanks of the preferred cleavage site, thereby identifying a preselected ribozyme. The specificity and effectiveness of the ribozymes, isolated from the bank and recloned, are determined by their incubation with the target RNA, as described below in Example 5.

Detailed Description Text (6):

The biological effectiveness, that is, the switching off of the function of the target RNA in vivo, is determined by transfection of the recloned ribozyme with the target gene in suitable cells, such as CHO, that means its employment as a stably expressing clone, and subsequent determination of the specific protein synthesis, such as hGH secretion as described below in Example 6. Further details of the invention are described in greater detail and are illustrated in the following examples.

Detailed Description Text (7):
The expression cassette is most suitably a vector for the antisense and for the ribozyme expression. This vector for antisense expression and ribozyme expression can bring about a continuous and stable expression of a particular desired ribozyme or an antisense sequence in a cell. This suitable expression cassette has a strong promoter, suitably a T7 promoter, an adenoviral va-RNA gene, a stable loop region, and an insertion site for the antisense/ribozyme sequence in the loop region.

Detailed Description Text (8):
The T7 promoter is suitably used in combination with T7 polymerase. The loop region is in a restriction site in the central part of the adenoviral va-RNA gene and its size is at least 2.times.21 bases of identical sequence. A suitable base sequence of the loop region is 5'-AACCCAGGTGTGCGACGTCAG-3' (Seq. Id. No. 10).

Detailed Description Text (10):
(A) the structure of the specific ribozyme for a 27 n.t. region about the GUC at position 988 within the exon IV of hGH RNA;

Detailed Description Text (11):
(B) the maps of plasmid matrices for ribozyme synthesis by in vitro transcription with pol III (HeLa extract) and T7 RNA polymerase; and

Detailed Description Text (13):
In an illustrative example of preparing this suitable expression cassette the T7Rz and T7Rzneo plasmids were linearized by a Hind III treatment. GvaRz and GvaLRz were used in circular form. hGH RNA was synthesized from a linear (SstI section) of genomic hGH gene (1663 nt) by in vitro transcription with T7 RNA polymerase (with 0.2 .mu.Ci.sup.32 P of CTP/.mu.g of RNA). An equimolar mixture (100 nM) of ribozyme and substrate was incubated at 37.degree. C. in 50 mM of Tris-HCl of pH 7.5 and 10 mM of magnesium chloride for 30 minutes with prior heat denaturation (90 seconds at 95.degree. C.). After the cleavage, the RNAs were purified and separated individually on a 6% polyacrylamide gel. Full-length RNA and ribozyme cleavage products (988 nt and 675 nt) were detected. The result shows that the embedding of the catalytic hammerhead structure in a stabilizing RNA (va) leads to a stable ribozyme, capable of functioning, only after the additional incorporation of the loop region.

Detailed Description Text (15):
Isolation of Specific Ribozymes From the Library

Detailed Description Text (16):
The strategy is shown diagrammatically in FIGS. 1 and 2. Synthetic ribozyme genes are prepared where the central part codes for the hammerhead ribozyme and both sides are flanked by random sequences (N.sub.11, N.sub.13) and a restriction site. The resulting fragment mixture was cloned into the GvaL cassette as an XhoI-NsiI fragment. A library of about 10.sup.9 different variants was created. The ribozymes were synthesized in vitro either by T-7 polymerase or po III of an HeLa extract using the library as the template. The RNA of CHO cells, which express the hGH gene steadily (5000 RNA copies per cell), was used as target sequence. Purified RNA was incubated with the in vitro transcribed ribozyme library. After purification of the cleavage products on an oligo-dT column, the 5' end of the downstream cleavage products was analyzed by means of the "RACE" technique as follows. After the reverse transcription with oligo-dT primers, the cDNAs were extended at the 3' end with dG, amplified with an oligo-dC and treated with hGH-specific primers, cloned into pGEMT (Promega) and sequenced. The sequences should start directly downstream from NUH identification sites (GUC, CUC) within the hGH RNA. The gene for the ribozyme, which brings about the cleavage of a selected site, was amplified by PCR from the ribozyme-plasmid library, specific, degenerate primers being used for the flanking regions of the ribozyme gene, as shown in the upper part of FIG. 3. After amplification, the resulting fragment was cloned

between the PstI and SalI sites of the vector GvaL. As shown at the bottom of FIG. 3, among the sequenced 50 clones, ribozymes with flanks of different length (from about 7 to about 13 nucleotides) were found for three ribozyme cleavage sites.

Detailed Description Text (18):
Cleavage of Cellular RNA by Transcribed Ribozymes

Detailed Description Text (20):
1./2. Purified total RNA (1 .mu.g/sample) as target. Ribozyme GvaLRz as T7 transcript; the in vitro transcript of GvaL served as control.

Detailed Description Text (21):
3. Cytoplasmic RNA/protein fraction as target. Cells (10.sup.5) were lysed in 50 mM Tris-HCl (pH 7.5) for 10 minutes in ice, frozen in liquid nitrogen and thawed at 37.degree. C.; the nuclei were then removed by centrifugation. A T7 transcript of GvaLRz (10 .mu.g) was used as ribozyme.

Detailed Description Text (22):
4. Cytoplasmic RNA as target. The ribozyme was prepared by pol III transcription (2 .mu.g/sample).

Detailed Description Text (25):
Specific in vitro Cleavage of hGH mRNA by Ribozymes From the Library

Detailed Description Text (26):
Total or cytoplasmic RNA preparations from hGH-producing cells are incubated with ribozymes from the library, which was transcribed either with pol III or T7 polymerase. hGH-specific 3' fragments are reversely transcribed and amplified by PCR. The PCR conditions are selected so that mainly fragments <1000 bp are formed. PCR products are separated on a 6% polyacrylamide gel; markers for the fragment size are applied on the left track. Six specific bands were found, the corresponding fragment lengths of which correlated with one in 4 exon sites (E1-E4) and 2 intron sites (I1, I2). It may be noted that T7 pol transcripts are more easily detected and that there are more cleavage sites in total RNA than in cytoplasmic RNA.

Detailed Description Text (28):
Localization of Ribozyme Cleavage Sites Within hGH mRNA

Detailed Description Text (33):
In vitro Cleavage of HGH-Specific RNA by Ribozymes From the Library

Detailed Description Text (34):
(A) Cleavage with 3 different, selected ribozymes. The ribozymes are transcribed with T7 polymerase from, in each case, a selected clone and incubated for 20 minutes at 37.degree. C. without heat denaturation with in vitro transcribed hGH RNA of the same molarity (both 100 nM). Samples are applied on a denatured 6% polyacrylamide gel. The resulting fragments have the expected size (E1: 1017, 646; II: 1099, 564; E5: 952, 711).

Detailed Description Text (35):
(B) Cleavage of hGH RNA by E1 ribozyme with complementary regions of different length. The incubation was carried out as in (A). The fragments were separated on a 4% denaturing polyacrylamide gel. As shown on the bottom of FIG. 3, the length of the complementary region of the E1 ribozyme is 26=13/13, 21=10/11 or 15=8/7. The two specific cleavage products can be detected only after incubation with ribozymes in the presence of magnesium. The most effective cleavage is found for the ribozyme with the shortest complementarily (15=8/7).

Detailed Description Text (37):
Effect of the Ribozyme Expression in vivo on the Level of the hGH Secretion

Detailed Description Text (38):
The transfection of CHO cells took place simultaneously with pCMVhGH, ribozymes or control constructs and pSV2neo. The short-term expression was tested after 3 days and the stable expression after 4 weeks after selection with geneticin. The hGH level was

determined with ELISA (limit of detection: 3 ng/mL). The level of hGH obtained with pCMVhGH+Gval was taken to be 100% (short-term: 7 .mu.g of hG/mL/24 hr; stable: 2 .mu.g of hGH/mL/24 hr). A mutant ribozyme (E1 8/7) was used as a control.

CLAIMS:

1. A ribozyme library comprising a collection of ribozyme genes encoding a hammerhead structure and flanking sequences of random nucleotides cloned at least once into an expression cassette for ribozyme expression, wherein said expression cassette contains a T7 promoter proximal to the 5' end of said cassette, an adenoviral va-RNA-gene adjacent to said promoter, and a loop region located in the central part of said gene, said loop region defined as a series of adjacent nucleotides between a first nucleotide and a second nucleotide, said first nucleotide further linked on either side to adjacent nucleotides other than the second nucleotide, and the second nucleotide further linked on either side to adjacent nucleotides other than the first nucleotide.

2. The ribozyme library of claim 1, wherein the library contains from about 10.sup.9 to about 10.sup.11 ribozyme genes.

3. The ribozyme library of claim 1, wherein said hammerhead structure comprises a double stranded DNA having the sequence CTGATGAGTCCGTGAGGACGAAAC (Seq. Id. No. 1).

4. A process for identifying and isolating a ribozyme, comprising

incubating with a ribozyme library a DNA or RNA sequence having a predetermined target sequence, said ribozyme library comprising a collection of ribozyme genes encoding a hammerhead structure and flanking sequences of random nucleotides cloned at least once into an expression cassette for ribozyme expression,

identifying the resulting cleaved targets, and

isolating the cleaving ribozyme.

6. The process of claim 5, wherein said process for identifying is carried out by a PCR reaction with gene-specific primers, and the isolation of the cleaved targets is carried out by electrophoresis in a gel.

7. The process of claim 5, wherein the ribozyme target RNA is isolated from the cleaved target gel, and identified by sequencing of the cleavage sites.

8. The process of claim 4, wherein a preselected ribozyme is isolated from the library by hybridization with two oligonucleotides that are specific for the preselected ribozyme, and the isolated ribozyme is employed as an expression clone.